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ATTORNEY'S DOCKET NUMBER
9013-42

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)
10/019198

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/GB00/02414	21 June 2000	21 June 1999

TITLE OF INVENTION
USE OF PEPTIDES

APPLICANT(S) FOR DO/EO/US
Proud et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4)
7. ☒ Amendments to the claims of the International Application Under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report Under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4)
20. ☐ Other items or information:

10019198 .061402

U.S. APPLICATION NO (if known, see 37 CFR 1.5) 10/019198	INTERNATIONAL APPLICATION NO PCT/GB00/02414	ATTORNEY DOCKET NO 9013-42

21. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492(a) (1) - (5)):	CALCULATIONS	PTO USE ONLY
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO..... **$1040.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO..... **$890.00**		
International preliminary examination fee 37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO..... **$740.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)..... **$710.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)..... **$100.00**		
ENTER APPROPRIATE BASE FEE AMOUNT =	$1,040.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	12- 20 =		x **$18.00**	$.00
Independent Claims	7- 3 =	4	x **$84.00**	$336.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$280.00**	$

TOTAL OF ABOVE CALCULATIONS =	$1376.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2 -	$	
SUBTOTAL =	$1,376.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).	$	
TOTAL NATIONAL FEE =	$	
Fee for Recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +	$	
TOTAL FEES ENCLOSED =	$1376.00	
	Amount to be refunded:	$
	charged:	$

a. ☐ A check in the amount of $.00 to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 50-0220 in the amount of $1376.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-0220. A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Karen A. Magri

Date: December 20, 2001

20792
PATENT TRADEMARK OFFICE

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Traci A. Brown

Attorney's Docket No. 9013-42

PATENT

IN THE UNITED STATES DESIGNATED OFFICE (DO/US)

In re: Proud et al.
International Application No.: PCT/GB00/02414
International Filing Date: June 21, 2000
For: USE OF PEPTIDES

Date: December 20, 2001

BOX PCT
Commissioner for Patents
Washington, DC 20231

PRELIMINARY AMENDMENT

Sir:

Please enter the following amendments prior to examining this application.

In the Specification:

Please make the following amendments to the specification:

On page one of the application, on a line below the Title of the Invention, insert the following:

- - **Related Application Information**

This application claims priority under 35 U.S.C. § 371 from PCT Application No. PCT/GB00/02414 (published under PCT Article 21(2) in English), filed on June 21, 2000, which claims the benefit of Great Britain Application Serial No. 9914480.0, filed on June 21, 1999, the disclosures of which are incorporated by reference herein in their entireties. - -

In the Claims:

Please cancel Claims 1-10 and insert the following new Claims 11-22:

11. (New) A method of inducing programmed cell death, said method comprising administering to a recipient a peptide comprising the sequence:

(K/R) xxYxxx(F/Q) L (L/M)

wherein x is a variable amino acid.

12. (New) A method according to claim 11 wherein said peptide comprises the sequence:

KKRYDREFLLGF,

RVRYSDQLLDL, or

RIIYDRKL (L/M).

13. (New) A method according to claim 11 wherein said peptide is 7-25 amino acids in length.

14. (New) A method according to claim 11 wherein said method induces cell death in tumour cells.

15. (New) A method of inducing cell death, said method comprising administering to a recipient a polynucleotide fragment encoding a peptide comprising the sequence:

(K/R) xxYxxx (F/Q) L (L/M)

wherein x is a variable amino acid.

16. (New) A method of inducing programmed cell death, said method comprising administering to a recipient a peptide of 7-25 amino acids in length comprising the sequence:

(K/R) xxYxxx (F/Q) L (L/M)

wherein x is a variable amino acid.

17. (New) A method of inducing programmed cell death in tumour cells, said method comprising administering to a recipient a peptide comprising the sequence:

(K/R) xxYxxx (F/Q) L (L/M)

wherein x is a variable amino acid.

18. (New) A method of inducing programmed cell death in tumour cells, said method comprising administering to a recipient a peptide of 7-25 amino acids in length comprising the sequence:

(K/R) xxYxxx (F/Q) L (L/M)

wherein x is a variable amino acid.

19. (New) A pharmaceutical formulation comprising a peptide comprising the sequence:

(K/R) xxYxxx (F/Q) L (L/M)

wherein x is a variable amino acid, and a pharmaceutically acceptable carrier.

20. (New) A pharmaceutical formulation according to claim 19 wherein said peptide comprises the sequence:

KKRYDREFLLGF,

RVRYSDQLLDL, or

RIIYDRKL (L/M).

21. (New) A pharmaceutical formulation according to claim 19 wherein said peptide is 7-25 amino acids in length.

22 (New) A pharmaceutical formulation comprising a peptide of 7-25 amino acids in length comprising the sequence:

(K/R) xxYxxx (F/Q) L (L/M)

wherein x is a variable amino acid, and a pharmaceutically acceptable carrier.

REMARKS

Claims 11-22 are pending in this application following entry of the present amendment. The specification has been amended to add a section entitled Related Application Information, which provides the priority claim and a statement regarding the publication of the international application in English. It is submitted that this application is now in condition for substantive examination, which action is respectfully requested.

Respectfully submitted,

Karen Magri

Karen A. Magri
Registration No. 41,965

Customer Number:

20792
PATENT TRADEMARK OFFICE

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Traci A. Brown
Date of Signature: December 20, 2001

12/pts

USE OF PEPTIDES

The present invention relates to eukaryotic Initiation Factor 4G (eIF4GI, GII) and derivatives of eIF4E Binding Proteins (4-E-BP1, 2, 3, 4) that interact with it.

By way of introduction, the proposed mechanism of eukaryotic initiation factor complex formation will be described with reference to Figure 1. The eIF4F complex is capable of initiating translation of 5' capped (m^7G) mRNAs[1]. This complex comprises eIF3, eIF4A, eIF4E and eIF4G (Figure 1).

- eIF4G acts as a scaffold around which the other components are assembled.

-eIF4A is a helicase which is required to unwind regions of secondary structure in the 5'UTR of the mRNA.

-eIF3 is responsible for recruiting the 40S ribosomal sub-unit to the complex, interacting with both the 40S ribosomal sub-unit and eIF4G.

eIF4E binds to both eIF4G and to the m^7G cap at the 5' end of the mRNA, hence recruiting the 40S ribosomal sub-unit to the 5' untranslated region (UTR) of capped mRNAs.

eIF4E independent routes exist for the initiation of translation of some messages[2] (eg. via an internal ribosome entry site (IRES)). However, mRNAs containing a long 5' UTR are dependent on eIF4E for the recruitment of the eIF4F complex to the m^7G cap, and the subsequent unwinding of the UTR by eIF4A. The critical role of eIF4E in cap dependent translation is attributed to the limited availability of the active species. eIF4E appears to be in limiting

amounts relative to other eIF4F components[1], requires phosphorylation (by Mnk 1[3]) for maximum activity and can be excluded from the eIF4F complex by binding to a 4E-BP[4,5] (Figure 2).

There is increasing evidence for a role of eIF4E in carcinogenesis. eIF4E induces cap dependent translation initiation in response to a number of mitogenic or proliferative stimuli[1,4,6]. Hormone and growth factor induced signal transduction can lead to hyperphosphorylation of 4E-BP by mTOR, resulting in the release of 4E-BP-bound eIF4E mTOR, resulting in the release of 4E-BP-bound eIF4E (Figure 2). Similar stimuli also lead to activation of eIF4E via phosphorylation by Mnk- 1. The resultant increase in eIF4E activity is required for the translation of several cap-dependent transcripts whose translation products are required for proliferation (eg. cyclin D1[7], Ornithine Decarboxylase (ODC)[3]).

The number of reports of increased levels of eIF4E in tumour samples is growing steadily[9,10], and in some cases eIF4E levels have been proposed to be a good indicator of prognosis[11,12]. Overexpression of eIF4E in cultured cell lines is reported to result in a transformed phenotype[13,14].

Overall these results have suggested that inhibiting eIF4E would result in inhibition of cap-dependent translation, resulting in little or no expression of mRNAs with strong eIF4E dependency for translation. This is expected to cause reduction in expression of several proteins involved in proliferation, and to reduce the

transformed phenotype of some tumour cells.

It has also been reported that overexpression of eIF4E is capable of acting as an anti-apoptotic survival signal in fibroblasts undergoing Myc-induced apoptosis in serum-restricted conditions[15].

The variety of eIF4E interacting proteins (eIF4G and 4E-BPs) has allowed identification of a common motif, (K/R)xxYDRxFL(L/M), required for binding to eIF4E[4]. Subsequently a 20 amino acid fragment of human 4E-BP1 containing this motif was shown to be capable of binding to recombinant mouse eIF4E and inhibiting cap-dependent translation in an *in vitro* translation assay[16], presumably by disrupting the formation of the eIF4F complex.

The proposed approach was to use eIF4E-binding peptides (derived from eIF4G and 4E-BPs) to inhibit formation of the eIF4F complex and reduce cap-dependent translation (Figure 3).

The present invention is based upon the observation that eIF4E binding peptides have been shown for the first time to induce programmed cell death. This observation is surprising given that the expected effect of such peptides was to reduce expression of several proteins involved in proliferation, resulting in growth inhibition of, or increased cytotoxicity to tumour cells. This surprising observation renders these peptides of utility in therapy.

Thus, in a first aspect the present invention provides the use of eIF4E binding agents, such as peptides or peptidemimetics in therapy, more particularly for the

induction of programmed cell death. Particular peptides found to be capable of inducing programmed cell death include a sequence of human eIF4G$_{569-580}$, wheat eIF4G$_{62-73}$ and human eIF4E-BP(1&2)$_{51-62}$ and derivatives and fragments thereof. Numbering according to Accession numbers AF104913, M95746, NM_004095 and NM_004096 respectively.

Thus the peptides of use in the present invention include the sequences;

human eIF4G$_{569-580}$, KKRYDREFLLGF

wheat eIF4G$_{62-73}$ RVRYSRDQLLDL and,

human eIF4E-BP(1&2)$_{51-60}$ RIIYDRKFL(L/M), and variants or derivatives thereof. A consensus may be derived from the above three sequences.

Thus, in a further aspect the present invention provides use of a peptide comprising a sequence:

YxxxxLØ

wherein x is a variable amino acid and Ø is Leu, Met or Phe;

or a fragment or derivative thereof in therapy, more particularly for the induction of programmed cell death.

Alternatively the peptide may comprise the sequence:

(K/R)xxYxxx(F/Q)L(L/M)

It is to be understood that "K/R" refers to an amino acid which is either lysine (K) or arginine (R), "x" may be any of the 20 amino acids or may be a synthetic or unnatural amino acid, "F/Q" refers to an amino acid which is either phenylalamine (F) or glutamine (Q) and "L/M" refers to an amino acid which is either leucine (L) or

methionine (M). The remainder of the sequence is understood to relate to the standard single letter symbol for amino acids.

Particular sequences may include KKRYDREFLLGF (human eIF4G$_{413-424}$), RVRYSRDQLLDL (wheat eIF4G$_{62-73}$) and RIIYDRKFL(L/M) (human eIF4E-BP$_{51-60}$).

The invention also relates to the use of fragments and derivatives of these peptides. Fragments are defined herein as any portion of the peptides described that substantially retain the activity of the parent peptide. Derivatives are defined as any modified forms of said peptides which also substantially retain the activity of the parent peptide. Such derivatives may take the form of amino acid substitutions which may be in the form of like for like eg. a polar amino acid residue for another polar residue or like for non-like eg. substitution of a polar amino acid residue for a non-polar residue as discussed in more detail below.

Thus, the present invention further provides derivatives of the sequences disclosed above for use in the induction of cell death.

Replacement amino acid residues may be selected from the residues of alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. The replacement amino acid residue

may additionally be selected from unnatural amino acids. Within the above definitions of the peptide carrier moieties of the present invention, the specific amino acid residues of the peptide may be modified in such a manner that retains their ability to induce programmed cell death, such modified peptides are referred to as "variants". Thus, homologous substitution may occur i.e. like-for-like substitution such as basic for basic, acidic for acidic, polar for polar, etc. Non-homologous substitution may also occur ie. from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (O), diaminobutyric acid (B), norleucine (N), pyriylalanine, thienylallanine, naphthylalanine and phenylglycine and the like. Within each peptide carrier moiety more than one amine acid residue may be modified at a time, but preferably when the replacing amino acid residue is alanine, less than 3.

As used herein, amino acids are classified according to the following classes;

basic; H,K,R

acidic; D,E

polar; A,F,G,I,L,M,P,V,W

non-polar; C,N,Q,S,T,Y,

(using the internationally accepted amino acid single letter codes)

and homologous and non-homologous substitution is defined using these classes. Thus, homologous substitution is used to refer to substitution from within the same class, whereas non-homologous substitution refers to substitution from a different class or by an unnatural amino acid.

In general, the term "peptide" refers to a molecular chain of amino acids with the defined biological activity. If required, it may be modified *in vivo* and/or *in vitro*, for example, by glycosylation, myristoylation, amidation, carboxybolation or phosphorylation. Thus inter alia peptides, oligopeptides and polypeptides are included. The peptides disclosed herein may be obtained, for example, by synthetic or recombinant techniques known in the art.

The term also extends to cover, for example, polypeptides which contain any of the above disclosed sequences and, in particular, wherein biological activity, that is, the polypeptide is capable of binding to eIF4E protein, is retained. Typically the length of the peptides of the present invention are between 7 - 25 amino acids in length, more preferably 10 - 20 amino acids in length.

In a further aspect the present invention provides use of a peptide comprising sequence:

YxxxxLØ wherein x is a variable amino acid and Ø is Leu, Met or Phe;

or fragment or derivate thereof in the manufacture of a medicament for therapy, more particularly for inducing cell death.

In particular, the peptide is used to induce the cell death in tumour cells.

In yet a further aspect, the present invention provides a polynucleotide fragment encoding a peptide comprising sequence:

YxxxxLØ wherein x is a variable amino acid and Ø is Leu, Met or Phe.

"Polynucleotide fragment" as used herein refers to polymeric form of nucleotides of any length, both to ribonucleic acid sequence and to deoxyribonucleic acid sequences. In principal, this term refers to the primary structure of the molecule, thus this term includes double stranded and single stranded DNA, as well as double and single stranded RNA, and modifications thereof.

As described above, the presence of a peptide comprising the above sequences can induce programmed cell death (apoptosis) in mammalian cells. The peptides of the present invention therefore have utility in treating diseases associated with undesirable cell proliferation/neoplasia. In particular the peptides have utility as anticancer or antitumour agents. Therefore, it may be desirable to direct the peptides to the site of action ie. the tumour. Thus, in the case of peptides, they may be conjugated to or associated with cell and/or tumour targeting agents, or in the case of the polynucleotide fragments provided as an expression cassette which comprises a polynucleotide sequence which encodes any of the above disclosed peptides, and a tumour-specific

inducible promoter which would allow expression of the peptide of the present invention only in tumour cells. The peptides of the present invention may also be conjugated or associated with agents designed to facilitate uptake into cell such as transport peptides eg. penetratin.

The present invention also relates to the use of peptidemimetics which bind eIF4E and function to induce programmed cell death. Such peptidemimetics are generally small molecules which function in the same manner as the peptides disclosed herein.

These and other aspects of the present invention will become apparent from the following description when taken in combination with the accompanying Figures, in which:

Figures 1 to 3 are diagrams illustrating the interaction of eIF4G and eIF4E;

Figure 4 illustrates the binding of human eIF4G-Penetratin conjugate to eIF4E and the sequences employed;

Figure 5 illustrates the binding of human, yeast, wheat and scrambled eIF4G, human 4E-BP1 and 4E-BP2 to eIF4E and the sequences employed;

Figure 6 illustrates human 4E-BP1 competing with eIF4G peptides for the binding of eIF4E and the sequences employed;

Figure 7 illustrates wheat $eIF4G_{(62-73)}$ inhibiting cap-dependent translation initiation;

Figure 8 is a table illustrating the induction of apoptosis by eIF4G peptides in MRC5 cells;

Figure 9 is a summary of the results of inhibition of $eIF4G_{(413-424)}$-induced apoptosis;

Figure 10 is a summary of the results of inhibition of $eIF4G_{(569-580)}$-induced apoptosis in MRC5 cells overexpressing constitutively active MEK/ERK;

Figure 11 is a diagram illustrating the interaction of eIF4G and eIF4E;

Figure 12 illustrates that eIF4E-binding peptides leads to rapid, dose-dependent, cell death. a). Sequences of biotinylated, penetratin-linked peptides. U=Norvaline, a substitution for cysteine. Conserved residues that are important in binding to eIF4E are underlined. b). *In vitro* binding assay. i) 1μg recombinant eIF4E was incubated with 0.2mM biotinylated peptides in a total volume of 50μl wash buffer (1xPBS/250mM Kcl) for 1h at 4°C. ii) 0.2mM Hu4G or Hu4G YLL-AAA was incubated with 200μg MRC5 cell lysate for 1h at 4°C in a total volume of 50μl wash buffer. In all cases the biotinylated peptides and associated proteins were pulled down using streptavidin agarose. The proteins were separated by SDS PAGE and subjected to Western blotting using anti-eIF4E antibody. Detection was by ECL;

Figures 13a-d are graphs showing cell survival (% of control, untreated cells) as measured by MTT assay. a) Lane 1: 10μM BP2 peptide added to serum-fed cells or Lane 2,3,4: 10μM BP2 peptide added to 24h, 48h or 72h serum-starved cells respectively. Lane 5: 10μM BP2 YLL-AAA added to 72h serum starved cells. Lane 6: 72h serum starved

cells incubated in 10% serum for 1h prior to addition to 10μM BP2 peptide. Lane 7: 72h serum starved cells incubated with 100μg/ml cycloheximide followed by a 1h incubation with 10% serum prior to the addition of 10μM BP2 peptide. In all cases the cells were then further incubated for 1h in 0.1mg/ml MTT. Cells were lysed in DMSO and absorbance was measured at 570mm. b-d) Varying concentrations of peptides were added to 72h serum starved MRC5 cells. After 30min incubation the cells were then further incubated for 1h in 0.1mg/ml MTT. Cells were lysed in DMSO and absorbance was measured at 570nm. All these results are representative of three separate experiments;

Figure 14 illustrates that cell death induced by eIF4E-binding peptides shows characteristics of apoptosis. a) Time lapse images of serum-starved MRC5 cells treated with 10μM Hu4G peptide for the indicated times. b) FACS analysis of MRC5 cells treated with 20μM Hu4G or Hu4G YLL-AAA for 40min. c) TUNEL analysis using the "In situ cell death detection kit" (Boehringer Mannheim). MRC5 cells were incubated with 10μM Hu4G peptide for 10min. Cells visualised with fluorescein by fluorescence microscopy. No signal was observed in untreated cells or cells treated with 10μM Hu4G YLL-AAA peptide (results not shown). 80% of cells incubated with the 10μM Hu4G peptide fluoresced positive. d) Images of DAPI-stained MRC5 cells incubated with either 20μM Hu4G or Hu4G YLL-AAA peptide for 40min. Arrows indicate the position of a cell with either a condensed nucleus (top right) or a nucleus with a punctate

appearance (bottom left). e) Visualisation of effects on the MPT. Serum-starved MRC5 cells were loaded with 0.1μM JC1 for 30min prior to addition of 10uM peptide. Changes in mitochondrial permeability were viewed by laser scanning confocal microscopy (krypton/argon laser) using identical setting. Green channel: excitation 488nm/emission 522nm. Red channel: excitation 568nm/emission 585nm. Channels were collected separately to avoid cross over. Images were taken 5min after addition of the peptide;

Figure 15 illustrates that the acute activation of MAP kinase protects cells from 4E-binding peptide induced cell death; and

Figure 16 illustrates that eIF4E binding peptide cell death is not through eIF4E's known role in mRNA translation. a) 72h serum-starved MRC5 cells were treated with 100μg/ml and 10μg/ml cylcoheximide, 1μg/ml and 0.1μg/ml pactamycine or 10μM Hu4G peptide for 2h prior to a MTT assay. Cells were pulsed labelled with [^{35}S]methionine for 30min after addition of the inhibitors/peptide. The incorporation of [^{35}S]methionine into protein was determined following hot trichloroacetic acid precipitation. b) 72h serum-starved MRC5 cells were treated with 100μg/ml cycloheximide (Chx), 1μg/ml pactamycine (Pact) or c) MRC5 cells which had been starved of serum for 72h were pre-incubated with 100μg/ml cycloheximide or 1μg/ml pactamycine for 30min prior to addition of 10μM Hu4G peptide. Cells were then further incubated for 1.5h prior to a MTT assay. Results from

three independent experiments (+/- SEM).

Examples

Abbreviations.

Amino acid and peptide nomenclature conforms to IUPAC-IUB rules (Eur. J. Biochem. 1984, 138, 9 - 37). Other abbreviations: Ahx, 6-aminohexanoyl; APase, alkaline phosphatase; DE MALDI-TOF MS, delayed-extraction matrix-assisted laser desorption ionisation time-of-flight mass spectrometry; DIEA, N,Ndiisopropylethylamine; PBS, phosphate-buffered saline (10 mM phosphate, 150 Mm NaCl, pH 7.4); PyBOP, Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid.

Material and Methods

General

The peptide deprotection/cleavage mixture used throughout was as follows: 0.75 : 0.5 : 0.5 : 0.25 : 10 (W/V/V/V/V) PhOH, H_2O, PhSMe, 1,2-ethanedithiol, TFA (Beavis, R.C., et al., (1992) Organic Mass Spectrometry 27, 156-158). Analytical and preparative RP-HPLC was performed using Vydac 218TP54 (4.6 x 250 mm) and 218TP1022 (22 x 250 mm) columns, respectively. Flow rates of 1 mL/min for analytical runs and 9 mL/min for preparative work were used (at 25°C). Gradient elution with increasing amounts of MeCN in H_2O (containing 0.1% TFA) over 20 min (anal.) and 40 min

(prep.) was performed. Eluants were monitored at X = 200 - 300 nm. Peptide samples were also analysed by DE MALDI-TOF mass spectrometry (ThermoBioAnalysis Dynamo instrument). An α-cyano-4-hydroxycinnamic acid matrix (Beavis, R.C. et al., (1992) Organic Mass Spectrometry 27, 156-158) was used and the appropriate m/z range was calibrated using authentic peptide standards in the m/z range 1,000 - 2,600.

Simultaneous multiple synthesis of peptides

Peptides were synthesisted using a Multipin Peptide Synthesis Kit (Chiron Technologies Pty. Ltd., Clayton, VIC, Australia). Peptide chains were assembled on "Macro Crowns" (SynPhase HM Series I, Rink Amide Linker; 5.3 μmol/crown) using Fmoc-amino acids (100 mM in DMF) and PyBOP/HOBt)/DIEA (1: 1: 1,5) coupling chemistry. The amino acid side-chain protecting groups were 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Arg), trityl (Asn and Gln) and t-butyloxycarbonyl (Lys and Trp). Activated amino acid solutions were dispensed using a PinAID device (Chiron Technologies). Coupling reactions were allowed to proceed for a minimum of 4h. All other chain assembly manipulations, including repetitive deprotection reactions (20% piperidine in DMF) and washing cycles (DMF and MeOH), were carried out according to procedures set out in the kit manual. After coupling and deprotection of the N-terminal βAla residues, (+)-biotin (300 mM in DMF) was coupled (chemistry as above for amino acids) during 4h. After washing and drying, the "Macro Crowns" were removed from

the synthesis device and placed into 10mL capped polypropylene tubes. To each tube was added 1.5 mL of cleavage/deprotection mixture. After 2h, the "Macro Crowns" were removed and washed with 0.5 mL each of neat TFA. To each tube containing the combined cleavage mixtures and washings Et_2O (8 mL) was added. After cooling to 4°C, the precipitated peptides were collected by centrifugation (4 min at 5,000 r.p.m.) and decantation. The pellets were resuspended in Et_2O (5 mL/tube). The suspensions were again cooled and the peptides isolated as before. The washing process was repeated once more before the crude peptides were dried *in vacuo*.

The crude peptides were redissolved in 0.1% aq TFA using sonication (2 mL/sample) and were applied to primed (MeOH then 0.1% aq TEA) solid-phase extraction cartridges (Merck LiChrolut RP-18, 500 mg). These were successively washed (2 x 2 mL 0.1% aq TFA each) and eluted (2 mL 0.1% TFA in 6 : 4 MeCN/H_2O). The eluates were evaporated to dryness by vacuum centrifugation.

Results and Discussion

The importance of eIF4E in translational regulation and cell growth is underscored by observations which show that overexpression of eIF4E leads both to increases in protein synthesis and to cellular transformation in human and mouse cells (17,18). The mechanism by which eIF4E overexpression leads to cell transformation is poorly understood. However, it is thought to be through the

elevated translation of growth related mRNAs, which are normally translationally repressed (19). In order to study directly the role of eIF4E in cell transformation, a series of experiments were carried out.

Human eIF4G$_{(413-424)}$ was conjugated to Penetratin, a known cell membrane translocation peptide of sequence RQIKIWFQNRRMKWKK (see patent EP485578). Description of its synthesis and coupling to other peptides may be found in US Patent 5,888,762. The human eIF4G$_{(413-424)}$-Penetratin conjugate was found to bind recombinant human eIF4E *in vitro* (see Figure 4). Surprisingly, wheat eIF4E$_{(62-73)}$ bound to and pulled down more recombinant human eIF4F *in vitro* than human eIF4G$_{(569-580)}$ did (see Figure 5). It was also observed that recombinant human 4E-BP1 competed with either human eIF4G$_{(569-580)}$ or wheat eIF4G$_{(62-73)}$ for binding of recombinant human eIF4E *in vitro* (see Figure 6).

Wheat eIF4G$_{(62-73)}$ was found to inhibit cap-dependent translation initiation, but not cap-independent translation initiation *in vitro* (see Figure 7). However, inhibition of cap dependent translation by eIF4G peptides was not detected in cultured mammalian cells. Furthermore, no inhibition of general translation by peptides from eIF4G or 4E-BP was detected in cultured mammalian cells.

Human eIF4G$_{(569-580)}$-Penetratin exhibited a cytotoxic or cytostatic effect on selected cell lines (HaCaT cells, no effect observed with short treatment (<24h with 20μM) but treatment of 60h serum starved cells began to die within 15 minutes of peptide treatment). Furthermore, human eIF4G$_{(413-}$

$_{424)}$-Penetratin and wheat eIF4G$_{(62-73)}$-Penetratin caused rapid cell death (possibly by apoptosis) in serum starved cell lines (see Figure 8).

Resistance to Human eIF4G$_{(569-580)}$-Penetratin and wheat eIF4G$_{(62-73)}$-Penetratin resulted from limited serum treatment (see Figures 9 & 2). However, this serum induced resistance could be inhibited by pre-treatment with MEK inhibitor PD 098059 (see Figures 9 & 2). Furthermore, serum induced resistance could be mimicked by the overexpression of a constitutively active MEK/ERK fusion (see figures 10 & 11). However, the serum induced resistance of cell lines was overcome using an increased concentration of peptide (72h serum-starved MRC5 cells died rapidly with addition of 10 μM 4G peptides; cells grown in 10% serum show similar biological effect with 40 μM 4G peptides; however, control peptides (triple Ala substitution) were not cytotoxic at 40 μM).

Conservation of structure activity relationship (SAR) was found in wheat and human 4G peptides and human 4E-BP peptides in binding assay, functional cell free assay and cell culture assays.

In order to study directly the role of eIF4E in cell transformation, a series of biotinylated synthetic peptides (Peptides synthesised by Cyclacel) corresponding to the eIF4E interacting domain (binding motif) of human eIF4G, and wheat eIF4G and alanine substituted peptides thereof were synthesised (see Figures 12a) and tested for their capacity to interact with ^{35}S-Met labelled *in vitro*

translated human eIF4E (NB. Peptides BP1 and BP1 YLM-AAA were not biotinylated). Peptides were coupled to streptavidin coated agarose beads by a N-terminus linked biotin group and washed in PBS/0.2% Tween 3x before being incubated for 1 hour at +4°C with *in vitro* translated human eIF4E. Beads were washed as above and boiled for 5 min. in SDS loading buffer before the peptide bound proteins were separated on a 10% SDS gel. The bands were visualised by autoradiography.

Triple alanine substituted derivatives such as Human $eIF4G_{(569-580)Y416AL421AL422A}$ (see Figure 8) were found not to inhibit cap-dependent translation initiation *in vitro*. However, 4G peptides containing specific single alanine substitutions (such as Human $eIF4G_{(569-580)Y416A)}$ partially inhibited cap-dependent translation initiation *in vitro*.

Triple alanine substituted derivatives such as Human $eIF4G_{(569-580)Y416AL421AL422A}$-Penetratin did not cause the observed biological effect (apoptosis) in MRC5 cells (see Figure 8). However, 4G peptides containing specific single alanine substitutions (such as Human $eIF4G_{(569-580)Y416A}$-Penetratin) had an intermediate biological effect on cultured mammalian cells, ie. reduced rate and extent of cell killing was observed.

The three different wild type peptides were shown to interact with human eIF4E and the H4G Y-A substitution had a lower binding affinity (see Figure 12b(i)). Scrambled human eIF4G peptide, the triple alanine human eIF4G peptide and the triple alanine wheat eIF4G peptides as well as the

single H4G L-A did not interact with eIF4E. The Hu4G peptide also bound eIF4E in cell lysates whereas the Hu4G YLL-AAA variant did not (see Figure 12b(ii)).

To investigate the effect of eIF4E binding peptides (eIF4G, BP1 and BP2) in living cells, 10μM of the eIF4E binding peptide BP2 was incubated with serum-deprived or serum-fed MRC5 cells. Un-expectedly, the addition of the BP2 peptide to 72h serum-starved MRC5 cells led to rapid cell death (within 1h) (Figure 13a, lane 4). In contrast, serum-fed cells were insensitive to the effect of the BP2 peptide at this concentration (Figure 13a, lane 1). Incubation of either serum-fed or serum-starved MRC5 cells with the triple alanine substitution peptide, BP2 YLL-AAA, had no significant effect on cell viability (Figure 13a, lane 5 and data not shown). The sensitivity of the cells to the effect of the peptide increased with the length of time the cells had been deprived of serum, with maximal effects observed by 72h serum starvation (Figure 13a, lanes 1-4). All subsequent experiments were therefore performed in cells deprived of serum for 72h. Re-addition of 10% serum for one hour to 72h serum-starved cells protected them from the effects of the peptide (Figure 13a, lane 6). This protective effect could not be inhibited by pre-incubation of the cells with cycloheximide, an inhibitor of general mRNA translation (Figure 13a, lane 7). This indicates that serum protects against peptide-induced cell death through a post-translational modification rather than by inducing synthesis of new proteins, e.g., cell survival

proteins. To investigate further the effect of eIF4E-binding peptides, serum-starved MRC5 cells were incubated with various concentrations of the BP2, BP1, Hu4G and W4G peptides (Figures 13b,c,d). Each of these peptides elicited rapid, dose-dependent, cell death (within 30 min using 20μM peptide and 1h for 10μM), whereas the addition of the triple alanine substitution peptides, which are unable to bind eIF4E, had no significant effect on cell viability (Figures 13b,c,d). The singly alanine-substituted peptide, Hu4G Y-A, which has reduced ability to bind eIF4E *in vitro,* also had a reduced capacity to induce cell death (Figure 13c). Another single alanine substitution peptide, Hu4G L-A, which was unable to bind eIF4E *in vitro*, had a severely reduced ability to induce cell death up to 10μM (Figure 13c). However, upon addition of higher concentrations (20μM), this peptide could induce cell death probably indicating some residual low binding affinity for eIF4E not detected in the pull-down assay (Figure 13c). All the peptides which are able to bind eIF4E *in vitro* can induce cell death even though they have very different sequences outside their common eIF4E-binding motif (Figure 12a). In addition, peptides harbouring single or triple alanine substitutions at conserved residues important in binding to eIF4E either had no significant effect on cell survival or a reduced ability to induce cell death. Taken together with the *in vitro* binding studies, these data underpin a strong structure/activity relationship and thus provide strong

evidence that eIF4E-binding peptides induce cell death through their interaction with eIF4E.

During eIF4E-binding peptide-induced cell death, cells shrank and underwent blebbing, two characteristics of apoptosis (Figure 14a). To investigate whether eIF4E-binding peptide-induced cell death also caused nuclear condensation and DNA cleavage, other characteristics associated with apoptosis, a number of methodologies were employed. FACS analysis of propidium iodide-stained MRC5 cells treated with the Hu4G peptide revealed a shift in the DNA profile from G0/1 to sub G0/1, indicating cell death and possible chromosomal DNA fragmentation/condensation (Figure 14b). DNA fragmentation was confirmed using TdT-mediated dUTP nick end labelling (TUNEL) (Figure 14c). Analysis of the cell nuclei by DAPI staining revealed that the cells incubated with the Hu4G presented signs of nuclear condensation, either having condensed nuclei or nuclei with a punctate appearance (Figure 14d).

An early event considered decisive in apoptosis is the opening of the mitochondrial permeability transition (MPT) pore (20-22). To characterise further the eIF4E-binding peptide-induced cell death, the development of the MPT was investigated in living MRC5 cells loaded with the fluorescent dye, JC1 (21). No changes in florescence were observed upon the addition of the inactive Hu4G YLL-AAA peptide (Figure 14e). In contrast, addition of the Hu4G peptide led to a rapid increase (within 5 min) in the intensity of the green fluorescence concomitantly with a

loss of orange fluorescence indicative of a drop in Ψm within the mitochondria due to the MPT pore opening (Figure 14e).

During apoptosis a conserved family of aspartic acid-specific cysteine proteases or caspases are frequently activated (23). However no such activation was detected in eIF4E-binding peptide-induced cell death (results not shown). Moreover, pre-treatment of the MRC5 cells with ZVAD.fmk, a wide spectrum caspase inhibitor, did not affect peptide-induced cell death (results not shown). Therefore, eIF4E-binding peptide-induced cell death appears not to involve caspase activation. Taken together, these data provide evidence that eIF4E peptide-induced cell death in MRC5 cells proceeds through a caspase-independent mechanism which exhibits a number of features observed in apoptosis. The rapidity with which the cells die and apparent lack of caspase activation are not features associated with classical apoptosis. However, it is clear that the activation of caspases is not a prerequisite for apoptosis (24,25). For example, it has been reported that mitochondrial associated protein, apoptosis inducing factor (AIF), can induce rapid caspase-independent apoptosis (26). The effect of these eIF4E-binding peptides on cell survival was also tested on a number of other cell-types including HaCaT, Swiss 3T3, RATI and IIeLa cells. In all of cases, addition of the Hu4G peptide resulted in rapid cell death whereas the Hu4G YLL-AAA peptide had no effect on cell survival (results not shown). However, characterisation of

the cell death process in these cells was not investigated in detail.

In the presence of 10% ECS, cells were resistant to treatment with 20μm of the peptides. Cells only died if they were serum deprived for 72 hours (more than 85%) within 15 minutes after the peptide had been applied (see Figure 15). However, if serum deprived cells (72h) were pre-treated with 10% FCS or with 20nM PMA (phorbol ester) for 15 minutes before the peptides were added, the cells survived the subsequent peptide treatment (60-70%).

Furthermore, if the serum deprived cells were instead pre-treated with the MAPK inhibitor PD098059 for 1 hour before 10% FCS was added, approximately 80-90% of the cells died. This result shows that cell death is linked to a genetic program and that the cells can be rescued from peptide induced death by addition of FCS or PMA. It is also suggested by the speed with which the cells died after peptide treatment and the rapid rescue by FCS or PMA and the effect of the MAPK inhibitor, that the effect of the peptides on cell death is dependent on secondary modifications in the cells.

Serum deprived cells were treated with the general translation inhibitors Cyclohexamide or Pactamycin at indicated concentrations or the H4G peptide in the presence of 35S-Met for 30 minutes (see Figures 16a,b). Cells were lysed and the amount of translation was estimated by counting incorporated 35S-Met in precipitated protein fractions. As expected, the peptide treated cells do not

incorporate 35S-Met and they die. However, general translation inhibitors block translation but they do not kill the cells.

It remained possible that eIF4E-binding peptide-induced cell death involved the up- or down-regulation of the translation of a specific mRNA or subset of mRNAs. To investigate this, MRC5 cells were treated with cycloheximide or pactamycin to prevent ongoing protein synthesis prior to the addition of the Hu4G peptide. However, this did not result in any protection against the effect of the Hu4G peptide (Figure 16c). These data show that continued translation is not required for the peptides to induce cell death, and thus provides evidence that the up- or down-regulation of the translation of a specific mRNA(s) does not mediate eIF4E-binding peptide-induced cell death.

In another experiment, cells that were serum deprived for 72 hours and pre-treated with general translation inhibitors were shown to be just as susceptible to cell death (85%) as cells not treated with translation inhibitors. This strongly indicates that the effect of cell killing by the peptides is <u>not</u> mediated by inhibition of translation and is therefore not mediated by a translation product. This observation is very surprising and novel.

The present data thus indicate that eIF4E plays a direct role in controlling cell survival that is not linked to its known role in regulating mRNA translation. It is

presently not clear what mechanism underlies this eIF4E-binding peptide-induced cell death. Without wishing to be bound by theory it is possible that it is associated with a yet undefined function of eIF4E. Recently, it has been reported that eIF4E co-localises in the nucleus with splicing factors and eIF4E may therefore play an additional role in splicing or RNA export (27). As penetratin-linked peptides can enter all compartments of the cell it is possible that these peptides interfere with a nuclear function of eIF4E which results in cell death. However, it is also possible that deleterious perturbations in eIF4E function may directly trigger the apoptotic machinery. This could be a "checkpoint" mechanism by which the cells sense the integrity of the translation machinery. Indeed, the rapidity of cell death suggests that binding of the peptides to eIF4E may directly signal the induction of cell death.

In conclusion, the present data clearly indicates that eIF4E plays a critical role in cell survival, which may be related to its known role in cell transformation. However, its role in cell survival appears to involve a novel mechanism independent of its known function in mRNA translation.

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CLAIMS

1. Use of an eIF4E binding agent in therapy.

2. Use according to claim 1 for the induction of programmed cell death.

3. Use according to either of claims 1 or 2 wherein the agent is a peptide or peptidemimetic.

4. Use according to claim 3 wherein said peptide comprises the sequence:
YxxxxLØ
wherein x is a variable amino acid and Ø is Leu, Met or Phe;
or a fragment or derivative thereof.

5. Use according to claim 4 wherein said peptide comprises the sequence:
(K/R)xxYxxx(F/Q)L(L/M).

6. Use according to claim 5 wherein said peptide comprises the sequence:
KKRYDREFLLGF,
RVRYSDQLLDL, or
RIIYDRKL(L/M).

7. Use according to any of claims 3 - 6 wherein said peptide is 7 - 25 amino acids in length.

8. Use of a peptide comprising sequence:
YxxxxLØ wherein x is a variable amino acid and Ø is Leu, Met or Phe;
or fragment or derivate thereof in the manufacture of a medicament for therapy, more particularly for inducing cell death.

9. Use according to claim 8 wherein the medicament is used to induce cell death in tumour cells.

10. Use of a polynucleotide fragment encoding a peptide comprising sequence:
YxxxxLØ wherein x is a variable amino acid and Ø is Leu, Met or Phe.

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WO 00/78803 A2

(54) Title: USE OF PEPTIDES

(57) Abstract: The present invention relates to use of eIF4E binding agents, more particularly peptides or peptidemimetics in therapy, such as induction of programmed cell death. Preferred peptides comprise the sequence YxxxxLØ wherein x is any amino acid and Ø is Leu, Met or Phe.

FIGURE 1

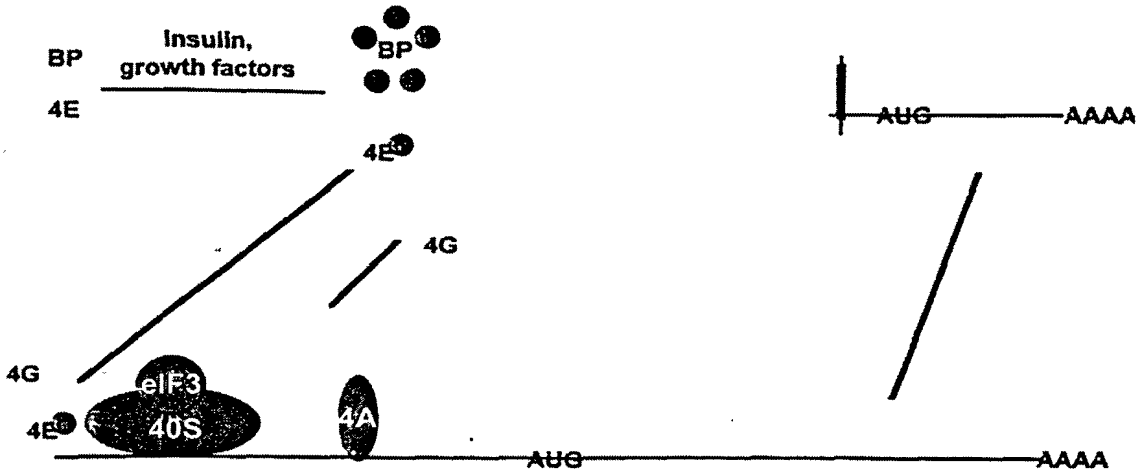


FIGURE 2

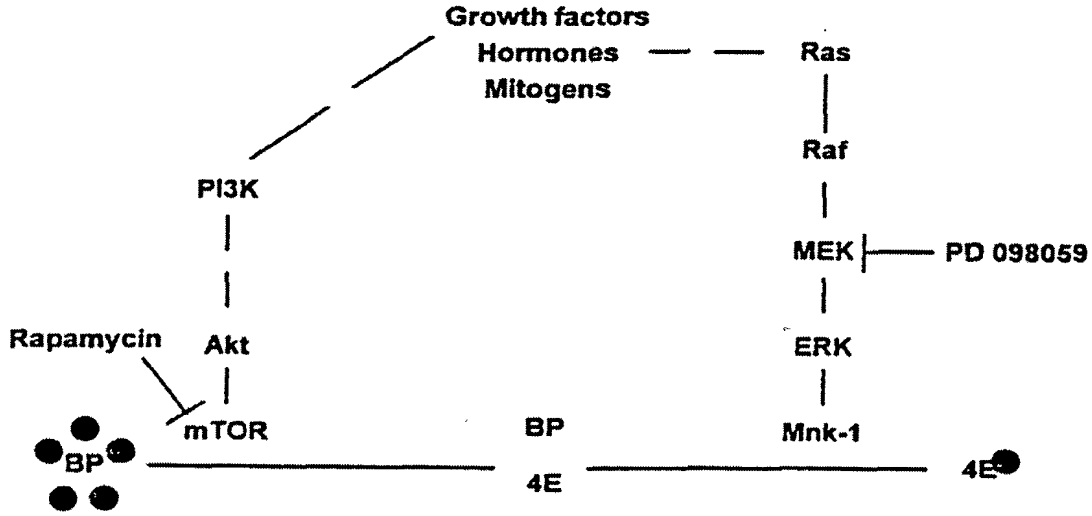


FIGURE 3

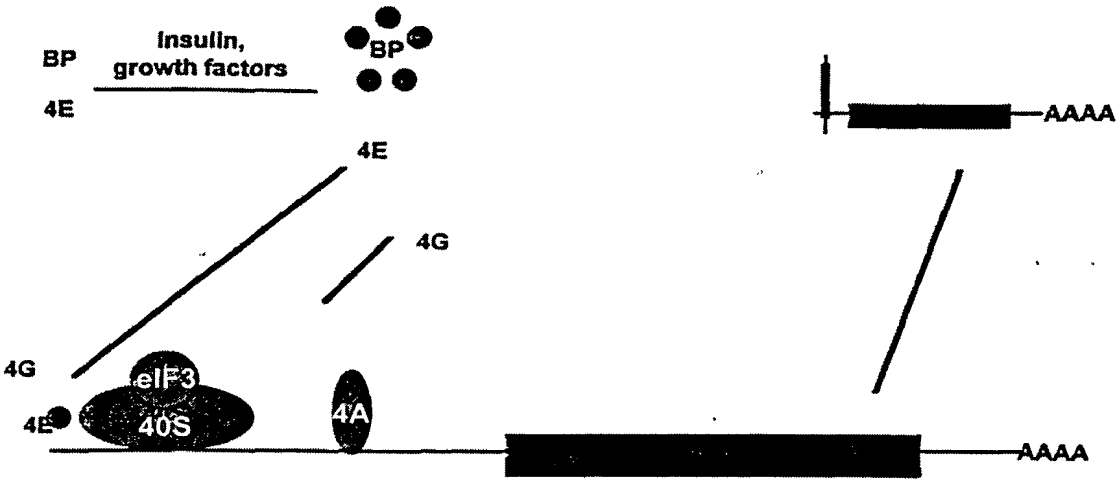
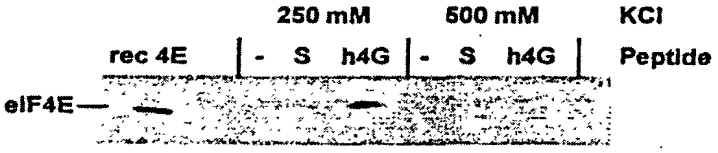


FIGURE 4



h4g human eIF4G B β KKRYDREFLLGFAARQIKIWFQNRRMKWKK

S scrambled eIF4G B β FDLKFALGRYRAEKRQIKIWFQNRRMKWKK

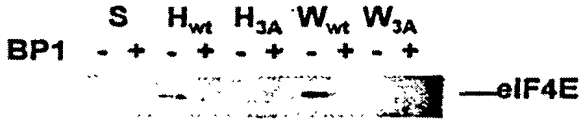
\- no peptide

rec 4E recombinant human eIF4E

FIGURE 5

H	human eIF4G	B	β	β	K	K	R	Y	D	R	E	F	L	L	G	F 413-424
Y	yeast eIF4G	B	β	β	K	Y	T	Y	G	P	T	F	L	L	Q	F 449-460
W	wheat eIF4G	B	β	β	R	V	R	Y	S	R	D	Q	L	L	D	L 62-73
1	human 4E-BP1	B	β	β	R	I	I	Y	D	R	K	F	L	M	E	U 51-62
2	human 4E-BP2	B	β	β	R	I	I	Y	D	R	K	F	L	L	D	R 51-62
S	scrambled eIF4G															

FIGURE 6



4G Peptide		Sequence												
H_{wt}	hu $4G_{(413-424)}$	K	K	R	Y	D	R	E	F	L	L	G	F	A A
H_{3A}	hu $4G_{(413-424)YALALA}$	K	K	R	A	D	R	E	F	A	A	G	F	A A
W_{wt}	wh $4G_{(62-73)}$	R	V	R	Y	S	R	D	Q	L	L	D	L	A A
W_{3A}	wh $4G_{(62-73)YALALA}$	R	V	R	A	S	R	D	Q	A	A	D	L	A A
S	scrambled hu 4G	F	D	L	K	F	A	L	G	R	Y	R	A	E K

all peptides biotinylated and linked to Penetratin™

FIGURE 7

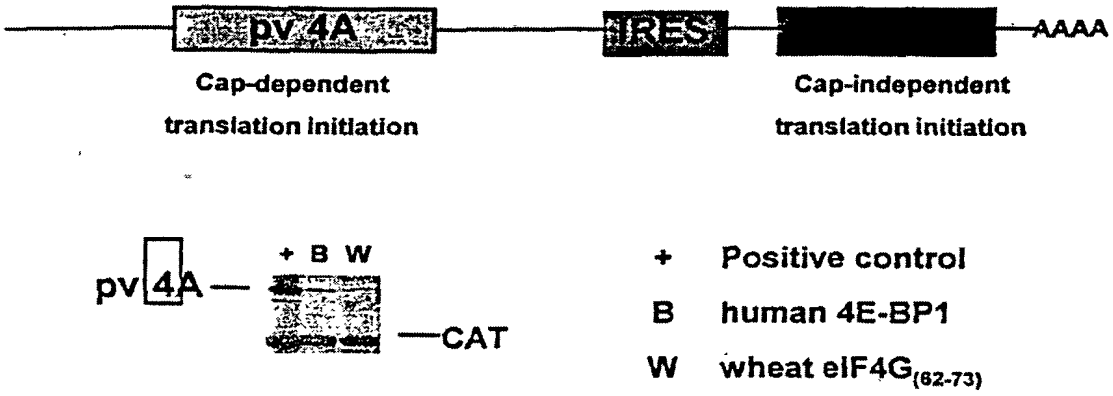


FIGURE 8

Peptide	Concentration			
	3 μM	6 μM	9 μM	12 μM
hu $4G_{(413-424)}$	+	++	+++	+++
hu $4G_{(413-424)Y416AL421AL422A}$	-	-	-	-
wh $4G_{(62-73)}$	-/+	+	++	+++
wh $4G_{(62-73)Y65AL70AL71A}$	-	-	-	-
scrambled hu 4G	-	-	-	-

MRC5 cells, 72 h serum-free growth, all peptides biotinylated and linked to Penetratin™

FIGURE 9

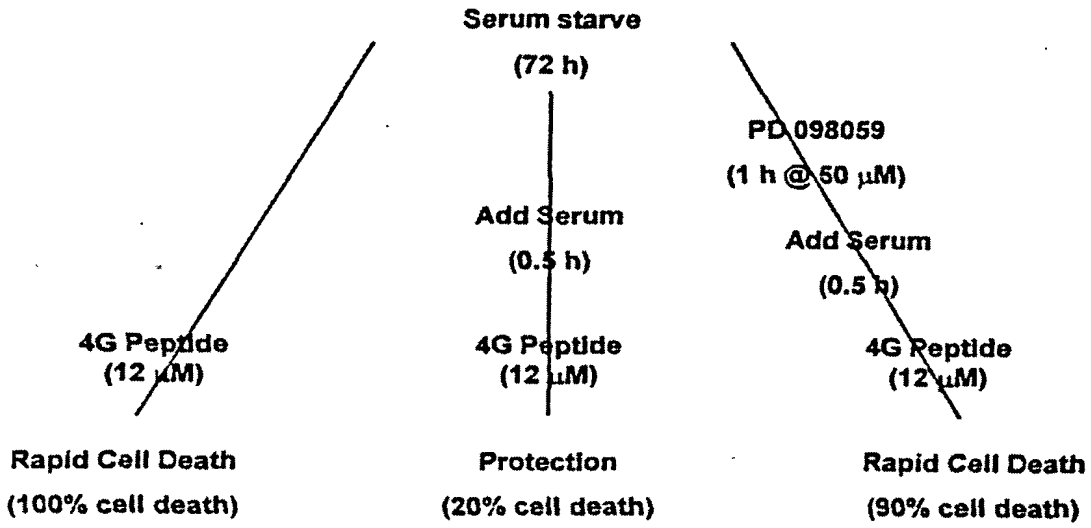


FIGURE 10

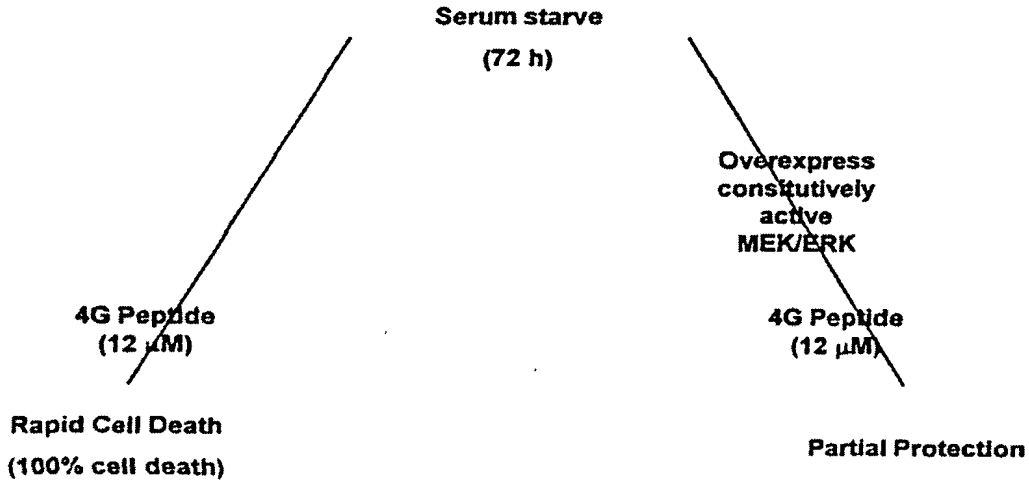


FIGURE 11

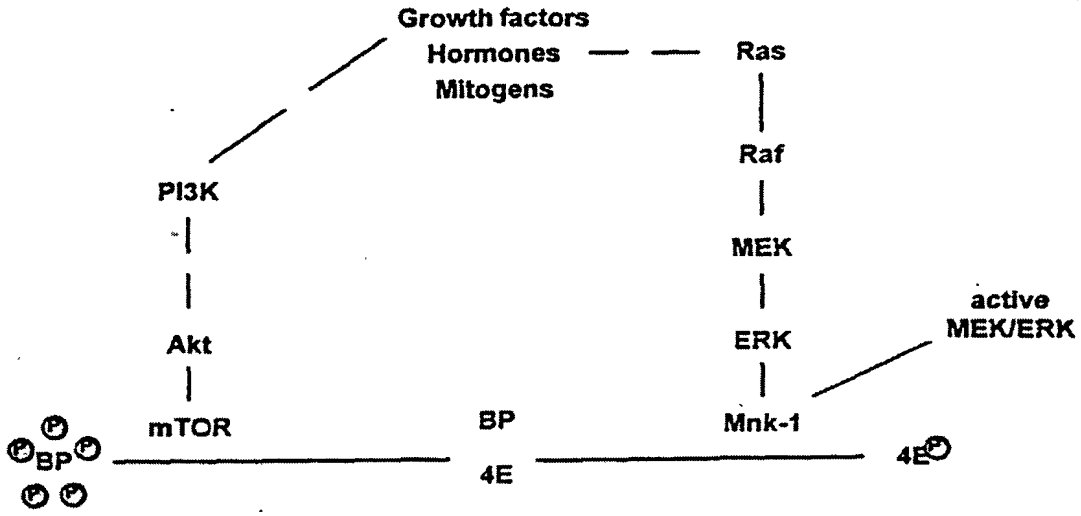

Growth factors
Hormones
Mitogens
Ras
Raf
MEK
ERK
active
MEK/ERK
PI3K
Akt
mTOR
BP
4E
BP
Mnk-1
4E

FIGURE 12

(a)

Hu 4G	Human eIF4G Peptide (569-580)Wild Type	KKRYDREFLLGF
Hu 4G YLL-AAA	Human eIF4G Peptide (569-580)Y572A L577A L578A	KKRADREFAAGF
Hu 4G Y-A	Human eIF4G Peptide (569-580)Y572A	KKRADREFLLGF
Hu 4G L-A	Human eIF4G Peptide (569-580)L577A	KKRYDREFALGF
W4G	Wheat eIF4G Peptide (62-73)Wild Type	RVRYSRDQLLDL
W4G YLL-AAA	Wheat eIF4G Peptide (62-73)Y65A, L70A, L71A	RVRASRDQAADL
BP2	Human 4E-BP2 Peptide (51-62)Wild Type	RIIYDRKFLLDR
BP2 YLL-AAA	Human 4E-BP2 Peptide (51-62)Y54A, L59A, L60A.	RIIADRKFAADR
BP1	Human 4E-BP1 Peptide (51-62)Wild Type	RIIYDRKFLMEU
BP1 YLM-AAA	Human 4E-BP1 Peptide (51-62)Y54A, L59A, M60A	RIIADRKFAAEU

(b)

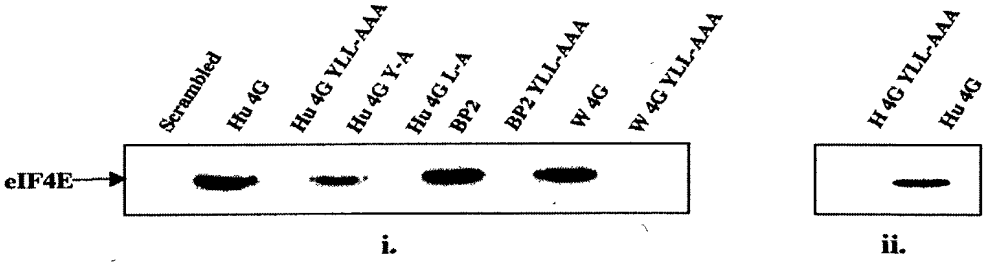
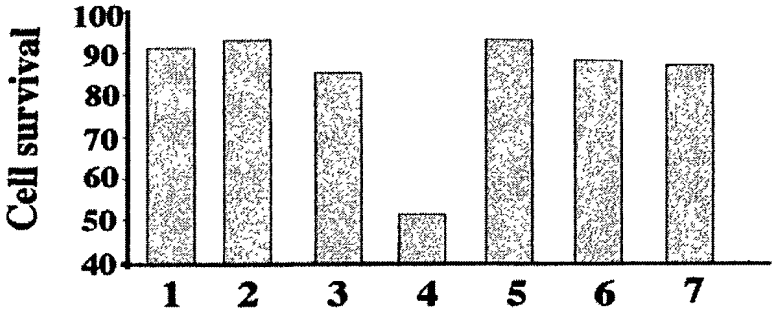
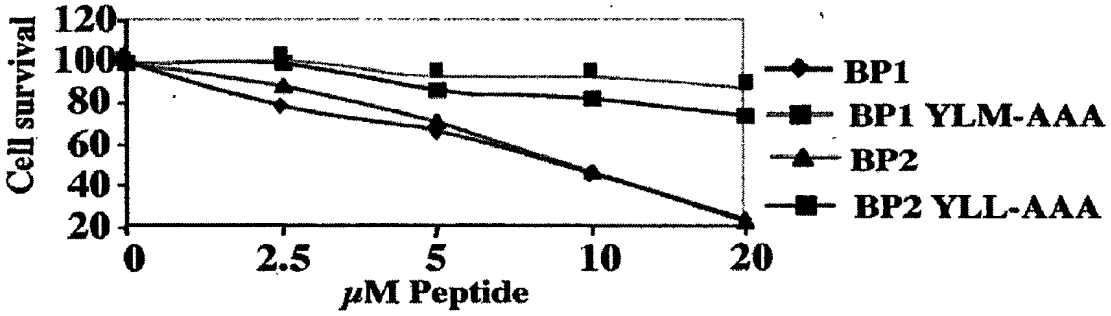


FIGURE 13

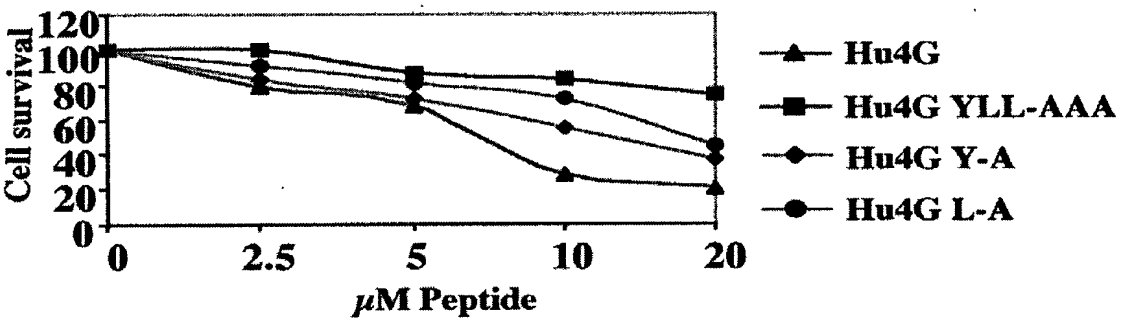
(a)



(b)



(c)



(d)

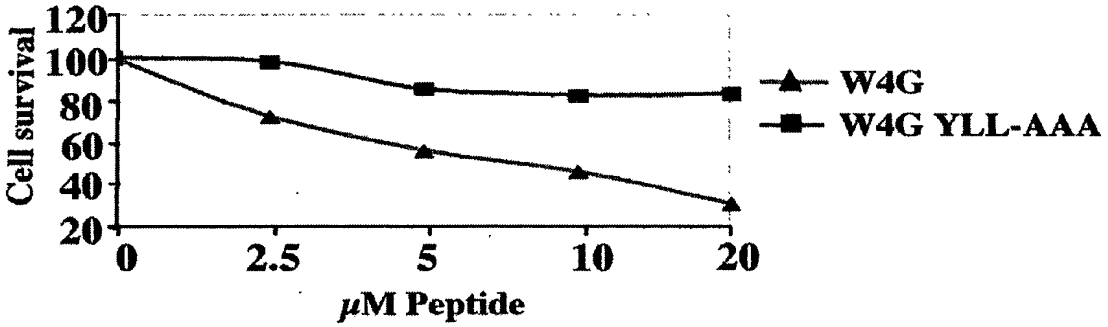


FIGURE 14

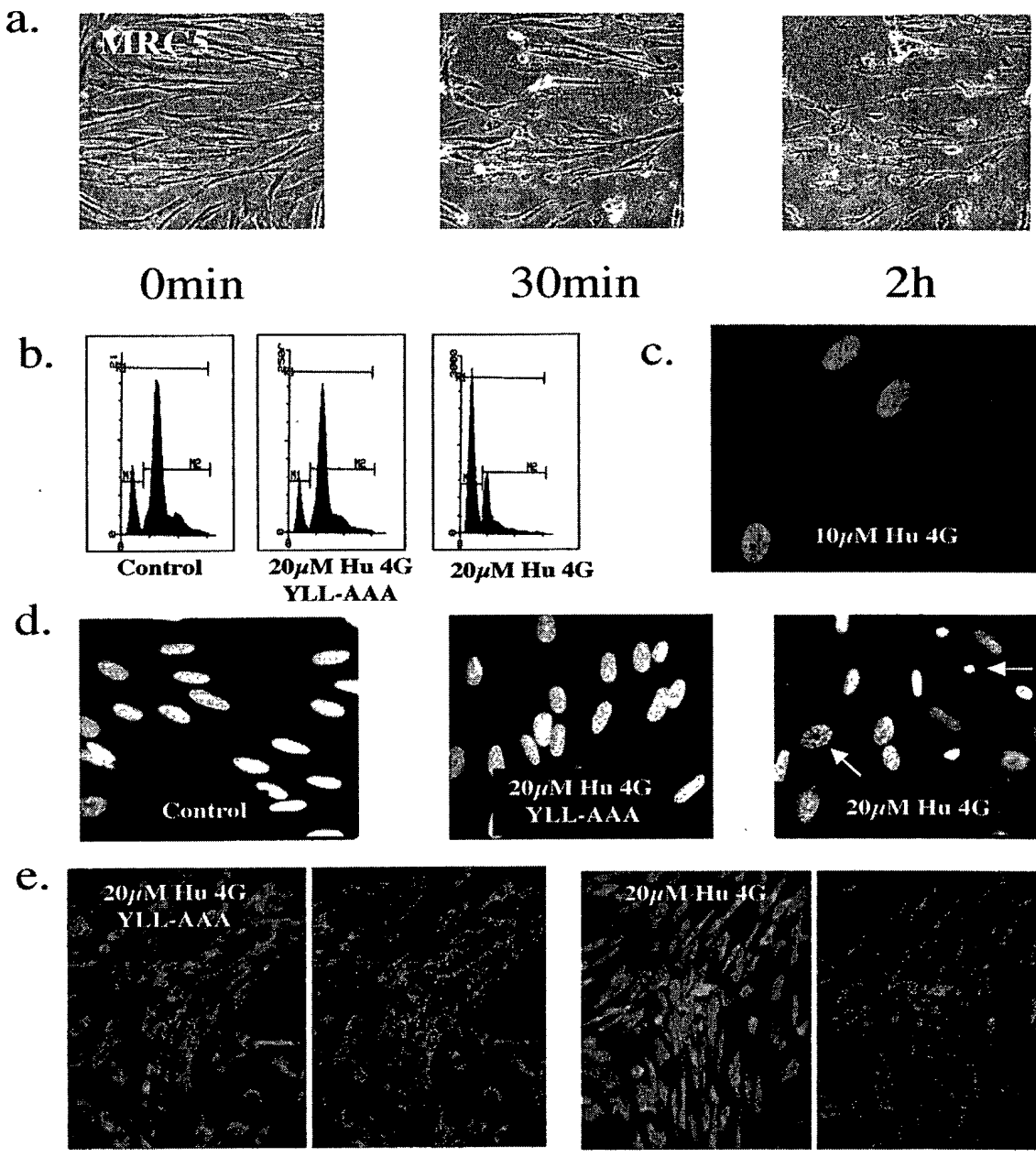

a.
MRC5
0min
30min
2h
b.
Control
20μM Hu 4G YLL-AAA
20μM Hu 4G
c.
10μM Hu 4G
d.
Control
20μM Hu 4G YLL-AAA
20μM Hu 4G
e.
20μM Hu 4G YLL-AAA
20μM Hu 4G

FIGURE 15

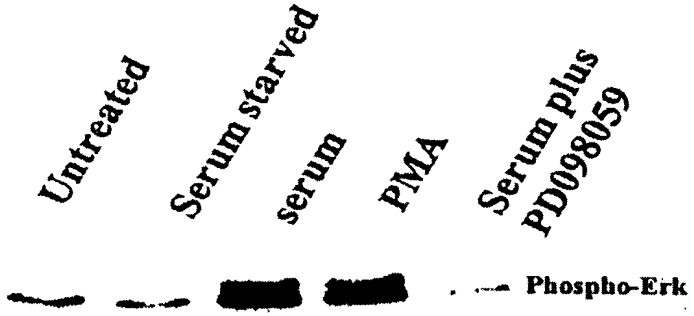

Untreated
Serum starved
serum
PMA
Serum plus
PD098059
Phospho-Erk

FIGURE 16

(a)

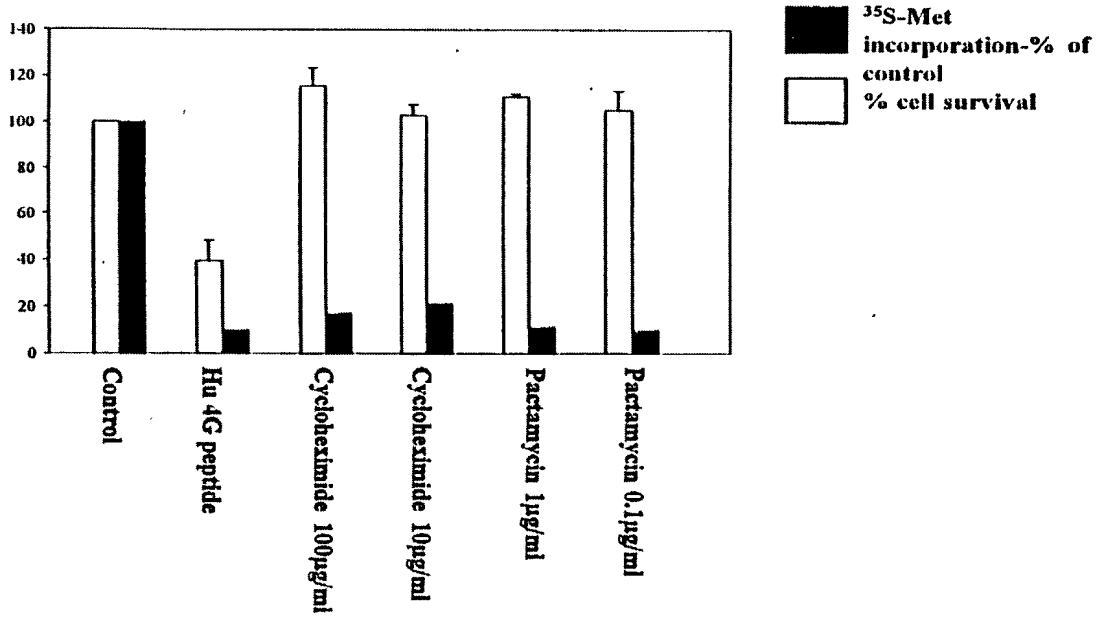

140
120
100
80
60
40
20
0
Control
Hu 4G peptide
Cycloheximide 100µg/ml
Cycloheximide 10µg/ml
Pactamycin 1µg/ml
Pactamycin 0.1µg/ml
35S-Met incorporation-% of control
% cell survival

FIGURE 16(cont.)